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GENETIC RECOMBINATION BETWEEN THE RESISTANCE TRANSFER FACTOR AND THE CHROMOSOME OF *ESCHERICHIA COLI*

HERBERT S. GINOZA AND ROBERT B. PAINTER

Exobiology Division, National Aeronautics and Space Administration, Ames Research Center, Moffett Field, California

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ABSTRACT

GINOZA, HERBERT S. (National Aeronautics and Space Administration, Moffett Field, Calif.), AND ROBERT B. PAINTER. Genetic recombination between the resistance transfer factor and the chromosome of *Escherichia coli*. J. Bacteriol. **87**:1339–1345. 1964.—Genetic instability for high-level streptomycin and chloramphenicol resistance was observed in several strains of *Escherichia coli* infected with the resistance transfer factor (RTF) episome. The altered site was always found on the chromosome, and the resistance characteristics were similar to, if not identical with, the corresponding determinant found on the episome. The high-level drug resistance phenotype was ascribed to two separate loci acting cooperatively within the host. The instability phenomenon had been attributed to a genetic exchange mechanism in which the chromosome copies the drug-resistance information from the episome, thus giving rise to a diploid homogenote for this segment. In a reciprocal exchange system, the tetracycline-resistance marker on the chromosome was shown to recombine with the RTF episome lacking this information.

Genetic instability in bacteria is commonly regulated by mutator genes residing on the chromosome (Treffers, Spinneli, and Belser, 1954; Miyake, 1960; Jyssum, 1960; Goldstein and Smoot, 1955). Some exceptional cases have been reported in which curable episome-type elements have been shown to dictate the rate of change of genetic information present on the bacterial genome. Gundersen, Jyssum, and Lie (1962) and Gundersen (1963) demonstrated that an infective factor originally found in a hospital strain of *Escherichia coli* enhances the mutation rate to streptomycin (Sm) resistance in many *E. coli* cultures harboring this particle. A related phenomenon was also reported for *E. coli* strains carrying the resistance transfer factor (RTF) episome [*see* Watanabe (1963) for a review of this multiple drug resistance factor] by Ginoza (1962) and Ginoza and Painter (1963). They have shown that an episome-infected culture exhibits several unusual characteristics: it mutates to high-level Sm and chloramphenicol (Cm) resistance at a much higher frequency than does the uninfected parental type; the mutated site is always present on the chromosome; the mutator effect disappears if the episome is removed with acridine dye; the mutagenic effect is limited to drug-resistance information already present on the episome; and the resistance characteristics of the mutated allele resemble the episomic information.

The present investigation was undertaken to determine whether the episome-induced mutations might occur through a genetic exchange system in which the chromosome copies part of the episomic information during replication. The probability that the RTF undergoes recombination with the host chromosome was implied by Watanabe and Lyang (1962).

MATERIALS AND METHODS

Cultures. A strain of *E. coli* B requiring thymine (*thy*) and proline (*pro*) for growth was supplied by R. Weatherwax. It was originally infected with an RTF episome lacking the tetracycline (Tc)-resistance gene. The drug-resistance level of this strain when tested on Penassay Base Agar (Difco) was: Sm, 25 μg/ml; Cm, 100 μg/ml; and sulfathiazole, 200 μg/ml. *E. coli* K-12 derivatives lacking the synthetic capacity for arginine (*arg*) and methionine (*met*), respectively, were obtained from T. Matney.

Media and drugs. Penassay Broth (Difco) was used routinely for growing liquid cultures and, in addition, for all the conjugation experiments. Drug-resistant mutants and episomic recipients were isolated on supplemented minimal medium (Davis and Mingioli, 1950) or on Penassay Base Agar to which the desired amount of antibiotics was added. Dihydrostreptomycin sulfate (Nutritional Biochemicals Corp., Cleveland, Ohio), tetracycline hydrochloride (Bristol Laboratories.

Inc., New York, N.Y.), chloramphenicol (Parke, Davis, & Co., Detroit, Mich.), and acridine orange (Schmid & Co., Stuttgart, Germany) were utilized as indicated.

Elimination of episome. Removal of the RTF was accomplished: (i) by the penicillin-screening technique of Watanabe and Fukasawa (1961); (ii) by growing a small inoculum (10^4/ml) in Penassay Broth containing acridine orange (20 μg/ml) at pH 7.6 (Hirota, 1960) for 24 hr, plating survivors on drug-free Penassay Base Agar, and then testing individual colonies on medium containing Sm and Cm for the presence of the RTF; and (iii) by the ultraviolet (UV)-acridine orange treatment of Watanabe and Fukasawa (1961).

Isolation of Tc-resistant mutants: *E. coli* K-12 F^- *met* strain is normally sensitive to Tc (1 μg/ml). Mutation to Tc resistance develops in a stepwise fashion. Resistant clones were isolated by plating appropriate dilutions of an overnight culture on Penassay Base Agar containing Tc (1 μg/ml). Mutant clones usually appeared after 72 hr of incubation. Resistant colonies were purified twice on the same drug-agar before subsequent use. Second and third step Tc-resistant mutants tolerating 2 to 5 and 10 to 25 μg/ml, respectively, were isolated in a manner similar to the first-step mutant.

Introduction of F factor. The fertility factor, F, was introduced into *E. coli* K-12 F^- *met* Tc_1r Tc_2r Tc_3r from *E. coli* K-12 F^+ *his* (histidine) by mixing equal volumes of exponential cultures (10^7/ml) and incubating statically at 37 C. F^+ recipients were selected on minimal medium containing methionine. The presence of the sex factor was further tested by spotting the suspected culture on a lawn of *E. coli* K-12 F^- *arg* overlaid on an unsupplemented minimal agar plate.

TABLE 1. *Occurrence of mutants resistant to high levels of streptomycin in RTF-infected cultures*

E. coli B strain	No. of cells plated	No. of streptomycin-resistant colonies
thy pro RTF........	4.6×10^8	3.5×10^4
thy pro (cured).....	2.2×10^8	0
thy pro (reinfected).	3.1×10^8	3.1×10^4

* Overnight cultures grown in Penassay Broth were serially diluted, and 0.1 ml of several appropriate dilutions was plated on the same agar medium containing dihydrostreptomycin (1 mg/ml).

Transmission of Tc resistance by the RTF and the F factors. Exponential cultures of *E. coli* K-12 F^- *met* Tc_1r Tc_2r Tc_3r (10^7/ml) infected with the RTF and the F factors, respectively, were mixed with an equal volume of *E. coli* K-12 F^- *arg* (10^9/ml). The mixtures were allowed to conjugate statically overnight at 37 C. Recipients receiving the Tc-resistance information were scored on a minimal medium supplemented with arginine and the selective antibiotic.

RESULTS

Occurrence of Sm-resistant mutants in RTF-infected cultures. When an overnight culture of *E. coli* B *thy pro* RTF was serially diluted and plated on Penassay Base Agar containing streptomycin (1 mg/ml), the number of resistant colonies recovered after 48 hr of incubation at 37 C was higher than expected (Table 1). The distribution of cells resistant to high levels of Sm remained constant in episomic cultures, regardless of the size of the starting inocula. Since the uninfected parental culture was not available for comparison of mutation frequency, the auxotrophic strain mentioned above was cured of its episome and tested for similar behavior. Unlike the RTF-containing strain, the cured strain did not appear to give rise to mutants resistant to streptomycin, even though a large population was screened. If a mutator factor is associated with the RTF episome, reinfection of the cured culture should restore the former activity. The data (Table 1) confirmed the prediction.

Site of mutational alteration. Chromosomal mutations conferring high-level resistance to Sm can occur either by a single gene alteration or through a series of changes involving several low-level-resistance loci. In a cell which contains the RTF factor, the possibilities are extended: the genetic change can occur exclusively within the confines of the episome itself; the mutated site can be restricted to the chromosome; or the modifications can occur simultaneously on both the chromosome and the exogenote. When *E. coli* B *thy pro* RTF that was resistant to high-level Sm was conjugated with an *E. coli* K-12 F^- *arg* recipient and plated on minimal agar containing arginine and Sm (500 μg/ml), no resistant colonies were observed, even after 72 hr of incubation. This medium was specifically designed to select episome recipients that had received the high-level Sm resistance locus. As a control, the conjugating mixture was also plated on the same medium containing lower levels of antibiotics (Sm, 25 μg/ml; Cm, 25 μg/ml). On the latter medium (used routinely for detection of the RTF episome) the number of recipients containing the episome with the unaltered drug-resistance information occurred as usual. These results indicated that the hereditary change did not occur on the episome. The logical alternative would then be that the mutation had taken place chromosomally. To show more precisely that the change had occurred on the chromosome, *E. coli* B *thy pro* RTF that was resistant to Sm (1 mg/ml) was cured of its episome by the UV-acridine orange technique. The presumably episome-free cell [based on the inability to transfer all or parts of the resistance factors and the failure to grow on agar containing Sm (25 μg/ml) plus Cm (25 μg/ml)] was tested for tolerance to various levels of Sm. It was found that without the episome the resistance level was only 25 μg/ml of Sm (on Penassay Base Agar). The result indicated that a low-level resistance alteration was induced on the host genome.

Mutation to high-level Cm resistance. Since the genetic analyses involving chromosomal or episomal transfer are more easily handled in the *E. coli* K-12 strains, all subsequent work was done with these organisms. *E. coli* K-12 F^- *met* strain infected with the RTF episome did not display the instability pattern for Sm resistance, but mutability to high-level Cm resistance was very obvious (Table 2). An uninfected control culture yielded the normal expected number of mutants. The mutation to Cm resistance in this strain occurs in a step-wise fashion. When a first-step Cm-resistant mutant was further assayed for high-level resistance mutants, only a few resistant colonies were recovered. The infected culture, on the other hand, gave rise to two distinct classes of mutants (Table 2). As expected, the frequency of mutants resistant to the intermediate level was slightly higher than the frequency of the more resistant type. To determine whether the mutated site had also occurred on the chromosome, a colony isolated from a plate containing 1 mg/ml of Cm was purified several times. (This technique allowed the isolation of a stable high-level resistant type.) Subsequently, it was grown in Penassay Broth and exposed to UV-acridine treatment. When 365 colonies were examined for elimination of the episome, 44 were found to be completely cured. The cured isolates were further examined for residual Cm resistance. Of 44 cured colonies, 34 were still resistant to 50 μg/ml; 3 were less resistant (10 μg/ml); and 7 were completely sensitive. These presumably cured isolates were unable to transfer the residual resistance locus when conjugated with competent recipients. On the other hand, they were still capable of readily accepting the RTF from various donor strains.

TABLE 2. *Mutation frequencies to chloramphenicol resistance in Escherichia coli K-12 F^- met strains*

Chloramphenicol	No. of resistant colonies		
	RTF-infected	Uninfected	First-step mutant*
μg/ml			
0	1.85×10^9	2.09×10^9	3.06×10^9
10	1.76×10^9	17	2.74×10^9
25	1.77×10^9	0	32
50	1.67×10^9	0	26
100	1.50×10^9	0	0
500	8.2×10^4	0	0
1,000	1.32×10^2	0	0

* First-step mutant resistant to Cm (10 μg/ml) was derived from uninfected culture. Overnight cultures grown at 37 C were decimally diluted and plated on Penassay Base Agar containing graded levels of antibiotics. Resistant colonies were scored after 72 hr of incubation.

Characteristics of the Cm-resistance locus in various mutants. Sm resistance induced by a cytoplasmic mutator (Gundersen, 1963) in *E. coli* differs from the ordinary single-step mutation. It occupies an area near the threonine locus on the chromosome and also acts as a suppressor of the ordinary *Sm* locus. Since the regular *Sm* locus in their system was unaffected, we decided to see whether the Cm resistance induced by the episome had some properties in common with the spontaneous mutant acquired from an uninfected culture. In this experiment the following resistant isolates were examined: (i) an RTF-infected culture that was resistant to Cm because of a resistant locus on the episome; (ii) an RTF-infected culture that had mutated to high-level Cm resistance, thus having two resistance loci, one on the episome and another on the chromosome; (iii) a high-level Cm-resistant mutant that was cured of its episome, thus retaining only the chromosomal locus; and (iv) a spontaneous first-step Cm-resistant mutant obtained from an uninfected culture. Approximately 100 cells from each culture were plated on Penassay Base Agar containing either Cm or Tc. The results (Table 3) clearly indicate that the Cm-resistance information on the episome differs from the resistance information acquired from the uninfected culture. The latter also carries information for Tc resistance. In addition, the data raise the possibility that the resistance information of the cured culture may have its origin on the episome.

Probable genetic recombination between the RTF and the chromosome. Genetic recombination in a partial diploid culture has been shown to occur quite frequently (Curtiss, 1962) because of the genetic homology between the chromosome and the exogenote. In the case of the RTF, it has been shown to contain two types of deoxyribonucleic acids (50 and 58% total guanosine plus cytosine content) when analyzed by the cesium chloride density-gradient technique (Falkow et al., 1963). Since *E. coli* also has a guanine-cytosine ratio of 50%, it appears feasible that some exchange can take place. Since the RTF episome in our system lacks the Tc-resistance information, it offered an excellent means to test the episome-chromosome genetic exchange hypothesis. As an initial starting point, an *E. coli* K-12 F^- *met* was made resistant to 25 μg/ml of Tc in three sequential step-wise mutations. This strain (labeled K-12 F^- *met* Tc_1r Tc_2r Tc_3r) was subsequently infected with the RTF episome lacking the Tc locus. According to our hypothesis, the Tc region on the chromosome may rarely undergo recombination with the episome, thus giving rise to an exogenote with the full resistance complement. To demonstrate that such a process can occur, this culture would have to potentiate the transfer of the recombinant episome in question to a suitable recipient. If a recipient is isolated which shows resistance to Sm, Cm, and Tc, then the existence of such a recombinant episome would be substantiated. Accordingly, the above Tc-resistant RTF-infected culture was used as a donor in several experiments to determine the validity of such a hypothesis. A typical experiment was conducted by mixing 10 ml of donor (10^7/ml) with an equal volume of recipient (*E. coli* K-12 F^- *arg*), approximately 10^9/ml. The cells were allowed to conjugate over-

TABLE 3. *Characteristics of chloramphenicol-resistance loci in episomic and nonepisomic Escherichia coli K-12 cultures*

Strain	No. of colonies	
	Cm (10 μg/ml)	Tc (1 μg/ml)
F^- *met* RTF	118	0
F^- *met* RTF *Cm-r**	97	0
F^- *met Cm-r* (RTF-cured)	102	0
F^- *met Cm-r†*	138	147

* High-level chloramphenicol-resistant mutant.
† First-step resistant mutant obtained from an uninfected culture.

TABLE 4. *Comparison of the RTF episome with the F episome in ability to transfer tetracycline resistance from Escherichia coli K-12 F^- met Tc_1r Tc_2r Tc_3r to E. coli K-12 F^- arg by conjugation**

Selective medium	To determine number of	Type of donor		
		RTF	F	Noninfected control
Min + met	Total donor	1.77×10^7	2.0×10^7	2.6×10^9
Min + arg	Total recipient	6.1×10^9	3.2×10^9	9.8×10^8
Min + arg + Tc	Recipient resistant to Tc	2.53×10^3	1.88×10^3	0
Min + arg + Sm + Cm	Recipient resistant to Sm and Cm	6.7×10^8	0	0
Min + arg + Sm + Cm + Tc	Recipient resistant to Sm, Cm, and Tc	1.98×10^3	0	0
Min + Tc	Revertant donor and chromosomal recombinant	0	0	0
Min	Revertant from donor or recipient and chromosomal recombinant	30	1.25×10^3	0

* Donors (RTF- and F-infected, 10^7/ml; control, 10^9/ml) and recipients (10^9/ml) mixed in equal volumes were allowed to conjugate statically overnight at 37 C. The mixtures were centrifuged, washed twice in 0.8% saline, and appropriate dilutions were plated on minimal medium supplemented as shown above. Min = minimal, arg = arginine, met = methionine, Tc = 5 μg/ml, Sm = 25 μg/ml, and Cm = 25 μg/ml.

night at 37 C to insure maximal episomic transfer. The mixture was subsequently washed in 0.8% saline, and appropriate samples were plated on various selective media designed to detect recipients that had received the various drug-resistance determinants. The data (Table 4) indicate that the RTF episome was capable of inducing the infected donor to transfer both the episomic characters and also the chromosomal Tc-resistance locus to the recipient during conjugation. The possibility that the Tc-resistance clones appeared because of a mutation of the donor to nutritional independence was ruled out, since no colonies were scored on minimal medium containing only Tc. The transfer mechanism is not known, but several possibilities will have to be considered: the *Tc* locus was copied on the RTF episome during replication, and was subsequently transferred as an integral part of this exogenote; the resistance information was introduced chromosomally; or the *Tc* gene was transferred nonspecifically (neither via episome nor chromosome). The third possibility can be discounted, since the transfer of the *Tc* locus was not detectable in the control experiment involving a noninfected donor (Table 4). Chromosomal transfer of the *Tc* locus cannot be excluded, but the result in Table 4 and other experiments to be described later suggest that our RTF episome does not initiate detectable transfer of other genetic markers during conjugation. The 30 prototrophic colonies in Table 4 are not recombinants but actually represent recipients that had reverted to nutritional independence. (In seven similar experiments, this colony type was not detected.) The recipient clones that had received the *Sm*, *Cm*, and *Tc* determinants were found to be genetically unstable, since they gave rise at a very high frequency to progeny that were sensitive to Tc. When these same resistant clones were tested as donors of the *Sm*, *Cm*, and *Tc* markers, they were found to be capable of transferring all three together but at a very low frequency. This observation tends to support the view that the *Tc* locus is associated with the episome.

The result of the Tc-resistance transfer in this type of experiment showed a great variation from no-transfer to approximately 1 per 10^4 donors. The factors influencing the efficiency of transfer are not known, but it was observed that the number of Tc-resistant recipients was always greater in experiments in which the episomal transfer was at its maximum.

Although these results strongly indicate that the Tc locus is transferred episomally, they do not completely eliminate the possibility of chromosomal transfer. Sugino and Hirota (1962) demonstrated that certain types of RTF can elicit chromosomal transfer. Our attempts to transfer various types of amino acid markers with the RTF were unsuccessful. With *E. coli* K-12 F^- *met* RTF as a donor and F^- *arg*, F^- *his*, F^- *try*, and F^- *ser* (derivatives of *E. coli* K-12) strains as recipients, the mating mixtures were allowed to conjugate for 24 hr in Penassay Broth. Then they were washed twice in saline, serially diluted, and plated on minimal agar. The results indicated that the RTF in our system is either incapable of or inefficient in mediating chromosomal transfer.

The F factor has been shown to transfer chromosomal information at a low frequency (Lederberg and Tatum, 1953). The chromosomal transfer induced by the RTF behaves similarly (Sugino and Hirota, 1962). To assess the possible role of chromosomal transfer in our system, an F factor-infected Tc-resistant donor was conjugated with a strain of *E. coli* not carrying this factor. The result in Table 4 indicates that the F factor induced the donor to transfer both the *Tc* and *met* loci at almost equal frequencies. On the other hand, the RTF was shown to be capable of initiating the transfer of only the *Tc* locus. These results assure the transfer system to be essentially independent of chromosomal transfer.

DISCUSSION

The present investigation reveals that some cytoplasmic elements are capable of unstabilizing chromosomal information. The mechanism involved in the instability process is not presently known, but Gundersen (1963) suggested that the cytoplasmic mutator (*Mu*) found in *E. coli* 635 *Mu* acts by attaching itself to a suppressor locus on the chromosome. The occupation by *Mu* of this site presumably interferes with the expression of the ordinary Sm-resistance locus. Since the RTF episome resembles the *Mu* factor in many details (they enhance mutation rate to streptomycin resistance; they do not themselves mutate, but induce the change on the chromosome; they can be removed from the host by acridine orange treatment; in their absence the host is unable to show the mutagenic property; they are both infectious), the possibility that such an association exists in our system has to be considered. Since the RTF episome disorganizes at least two loci

(*Sm* and *Cm*), not simultaneously but each at a specific rate, it does not seem that a suppressor attachment system is operative in the RTF-infected cells.

The result with the high-level Sm-resistant mutant discloses that the mutational change actually occurs on the chromosome. It is not an alteration that confers a high-level resistance, but a low-level one similar, if not identical, to the *Sm* locus on the episome. Whether the altered locus is allelic to the episome *Sm* locus is still not known. Until a genetic analysis of the modified chromosome is made by sexual recombination experiments, the nature of the *Sm* genotype induced by the episome will not be known.

Genetic exchange between episome and chromosome has been recently reported by Falkow and Baron (1962) in *Salmonella typhosa* ST-2, which carries the episome, F_0 *lac*$^+$. The *lac*$^+$ region on this episome has been shown to undergo a nonreciprocal recombination with the chromosome, thus resulting in a cell with *lac*$^-$ homogenote. In addition, a rare isolate devoid of the episome but showing a *lac*$^+$ genotype was also recovered from the same culture. These illustrations strongly suggest that there is a definite relationship between the various mutational events occurring in episomic cultures and the recombinational potential between the chromosome and the exogenote. Our results with the Cm-resistant mutant arising from the episome-infected culture imply this type of genetic recombination. When a highly Cm-resistant culture was cured of its episome, several Cm-resistant types were found. Most were resistant to 50 μg/ml, some to 10 μg/ml, and others were completely sensitive. The probability that a chromosomal mutation of 50 μg/ml will occur in a single-step mutation is very remote, since it involves at least two stepwise mutations, each at a frequency of 10^{-8}. In addition, the mutation to Cm resistance in a noninfected type differs in one very important respect from the episome-induced type. The former always confers, at the same time, resistance to Tc, but the episome-induced type does not. These results indicate (but do not prove) that Cm-resistant mutants in RTF-infected cells arise by a copy-choice phenomenon similar to the episome-chromosome exchange system of Falkow and Baron (1962). Our data also resemble theirs in the frequency of probable recombination (1 per 10^5 divisions).

Watanabe and Lyang (1962) postulated that the unusually high rate of segregation of drug-resistance markers in an *S. typhimurium* RTF is due to a genetic exchange between the sensitive alleles on the chromosome and the resistance sites on the episome. Our findings with the Tc-resistant mutant infected with the RTF support such a hypothesis. In this culture, the Tc-resistance sites were located on the chromosome and, since the RTF episome was lacking this factor, the two possible ways in which the *Tc* genes could have been transmitted to the recipient during conjugation were either through chromosomal transfer or by being associated with the episome. Sugino and Hirota (1962) demonstrated that some RTF are capable of inducing chromosomal transfer in *E. coli*. This possibility cannot be entirely excluded, but in several experiments we have been unable to detect any such event; chromosomal transfer, if it does occur, is of such a low magnitude that it cannot account for any appreciable fraction of the observed transfer. Moreover, if the RTF used in our system is capable of chromosomal transfer, we should have observed transmission of other genetic determinants, such as those mediated by the F factor in the same host. Therefore, our data indicate that the chromosomal Tc-resistance gene is copied on the RTF episome and is transmitted as a single linkage group during conjugation.

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